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(71) Applicant *(for all designated States except US)*: TEMPLE UNIVERSITY – OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; Broad Street and Montgomery Avenue, Philadelphia, PA 19122 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: MOSSER, David, M. [US/US]; Apartment E3, 209 N. 4th Street, Philadelphia, PA 19106 (US).

(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, PA 19102 (US).

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(54) Title: GENETICALLY ENGINEERED RHODOCOCCUS VACCINE

(57) Abstract

The invention provides methods and compositions for immunizing a vertebrate against Rhodoccocus infection and for preventing the pneumonia associated with *R. equi* infection. The invention also provides a method for expressing recombinant nucleic acids in Rhodococcus cells.

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GENETICALLY ENGINEERED RHODOCOCCUS VACCINE

Cross-reference to Related Applications

This application claims the benefit of United States Provisional Application Serial No. 60/053,937, filed 28 July 1997.

Field of the Invention

This invention relates to methods and compositions for immunizing a vertebrate against Rhodococcus infection, and to methods for expressing recombinant nucleic acids in Rhodococcus.

Background of the Invention

A) *Rhodococcus equi*

Members of the genus Rhodococcus are gram-positive, aerobic, non-sporulating, partially acid-fast Actinomycetes, which were formerly classified as Nocardia, Mycobacterium, Gordona, Jensenia, or in the "rhodochrous" complex. Nocardia, Corynebacteria and Mycobacterium are closely related to Rhodococcus, each exhibiting nocardioform morphology, having mycolic acids, meso-diaminopimelic acid, arabinose and galactose in their cell walls and having a high G+C content (>59 mol %) in their cellular DNA. Rhodococcus species are common in soil, and inhalation of dust is believed to be a primary mechanism of infection in both animals and man. Several pathogenic species exist, including *R. fascians*, a plant pathogen, *R. bronchialis*, a human pathogen, and *R. equi*, an animal pathogen.

Rhodococcus equi (*R. equi*, formerly called *Corynebacterium equi*) is a facultative intracellular bacterium that is one of the leading causes of pneumonia in young horses (foals).

Rhodococcus equi, which is endemic on some horse farms, can cause extensive morbidity and mortality. Rhodococcal disease occurs primarily in foals under six months of age, with the majority of cases occurring in foals under three months of age. *R. equi* generally does not cause pneumonia in older horses because they are able to mount an effective immune response, but the organism can cause disease in older animals that are immunocompromised or have other systemic illnesses. At one veterinary institution, *R. equi* infection accounted for 10% of all foals submitted for postmortem, and 40% of foals with pneumonia (Zink *et al.*, *Can. Vet. J.* **27**:213 (1986)). Disease caused by *R. equi* can have a considerable negative economic impact on the equine industry, resulting from the high cost of therapy and the loss of the athletic potential of both deceased and surviving horses. Infection with the bacterium can result in a severe, often life-threatening pneumonia (Prescott, *Clinical Microbiology Reviews* **4**:20-34 (1991)). Foals infected with *R. equi* may also develop ulcerative colitis, mesenteric lymphadenitis, septic arthritis, serositis, intervertebral abscessation, or cutaneous ulcerative lymphangitis.

In the lungs, pulmonary macrophages phagocytize *R. equi* and become the site for bacterial replication *in situ*. Intact, viable bacteria are found within macrophages obtained by alveolar lavage. *R. equi* pneumonia can be insidious in nature, and consequently is often quite advanced at the time of clinical recognition. Because of its insidious nature, treatment of the pneumonia typically necessitates long term antimicrobial therapy which is very costly and not always effective. Even when the therapy is effective, protracted pulmonary disease can affect the eventual performance of the adult horse.

R. equi also causes chronic cervical lymphadenitis in swine.

Although *R. equi* is not generally considered pathogenic in humans, it can cause infection and even death in AIDS patients and in individuals who are otherwise immunocompromised. The primary manifestation of *R. equi* infection in humans, as in foals, is pneumonia.

B) Virulence of *R. equi*

In examining the protein profiles of different clinical isolates of *R. equi*, Chirino-Trejo and Prescott noted the presence of a diffuse 17.5 kDa protein, the expression of which was observed to be temperature-dependent and lost upon repeated passage in culture (Chirino-Trejo and Prescott, *Canadian Journal of Veterinary Research* **51**:297-300 (1987)). Later, Takai and colleagues showed that all isolates which were virulent for mice expressed major protein antigens of 15-17 kDa (Takai *et al.*, *J. Clin. Micro.* **29**:439-443 (1991)). Takai *et al.* further demonstrated that sera obtained from foals naturally infected with *R. equi* consistently reacted with the 15-17 kDa protein antigens, whereas sera from healthy foals did not. Shortly thereafter, two groups reported the plasmid profiles of different strains of *R. equi* isolated from animals with clinical disease. All strains that expressed the 15-17 kDa antigens contained a large plasmid of approximately 85 kb (Takai *et al.*, *Infect. and Immun.* **59**:4056-4060 (1991); Tkachuk-Saad and Prescott, *J. Clin. Microbiol.* **29**:2696-2700 (1991)). The 15-17 kDa antigens were shown to be thermoregulated and apparently surface-expressed (Takai *et al.*, *Infect. Immun.* **60**:2995-97 (1992)).

Using SDS-PAGE, Tan *et al.* demonstrated that *R. equi* produce three antigenically-related virulence-associated proteins: a diffuse 18-22 kDa protein, a 17.5 kDa protein, and a 15 kDa protein (*Can. J. Vet. Res.* **59**:51-59 (1995)). A single gene was cloned and shown to encode all three proteins, the molecular weight differences apparently being due to lipid modification. This gene was designated *vapA*, its protein product was designated VapA. Immunization of mice with the lipid-modified VapA purified by SDS-PAGE fractionation or with acetone precipitated VapA protein following TX-114 extraction resulted in enhanced clearance from the liver and spleen following intravenous challenge. The VapA protein was therefore hypothesized to be a protective immunogen.

C) Immunization

The immune system of vertebrates consists of several interacting components. The best characterized and most important parts are the humoral and cellular (cytolytic) branches. Humoral immunity involves antibodies, proteins which are secreted into the body fluids and which directly recognize an antigen. The cellular system, in contrast, relies on special cells which recognize and kill other cells which are producing foreign antigens. This basic functional division reflects two different strategies of immune defense. Humoral immunity is mainly directed at antigens which are exogenous to the animal whereas the cellular system responds to antigens which are actively synthesized within the animal.

Antibody molecules, the effectors of humoral immunity, are secreted by special B lymphoid cells, B cells, in response to antigen. Antibodies can bind to and inactivate antigen directly (neutralizing antibodies) or can activate other cells of the immune system to destroy the antigen.

Cellular immune recognition is mediated by a special class of lymphoid cells, the cytotoxic T lymphocytes (CTLs). These cells do not recognize whole antigens but instead respond to degraded peptide fragments thereof, which appear on the surface of the target cell bound to proteins called class I major histocompatibility complex (MHC) molecules. Essentially all nucleated cells have class I molecules. It is believed that proteins produced within the cell are continually degraded to peptides as part of normal cellular metabolism. These fragments are bound to the MHC molecules and transported to the cell surface. The cellular immune system constantly monitors the spectra of proteins produced in all cells in the body and is poised to eliminate any cells producing foreign antigens. The cellular immune response also involves helper T cells which produce cytokines and thereby elicit participation of additional immune system cells.

Immunization, which comprises the administration of a vaccine in order to induce an immune response, is a method for preparing an animal to

respond to an antigen. Immunization is more complex than immune recognition and involves not only B cells and cytotoxic T cells but other types of lymphoid cells as well. During immunization, cells which recognize the antigen (B cells or cytotoxic T cells) are clonally expanded. In addition, the population of ancillary cells (helper T cells) specific for the antigen increases. Immunization also involves specialized antigen presenting cells which can process an antigen and display it in a form which can stimulate one of the pathways of the immune system.

Immunization continues to be the best way to prevent infectious diseases, and the development of new vaccines has a profound effect on both human and animal health. The first successful vaccine by Jenner, developed almost two hundred years ago, has resulted in the eradication of small pox disease from the planet. The present decade alone has seen the introduction of several new vaccines, and children now routinely receive vaccinations for diphtheria, tetanus, pertussis, measles, mumps, and even H. influenza.

Macrophages are active secretory cells which can exert an influence on the developing immune response by producing immunomodulatory cytokines. One of the important cytokines that is made by macrophages is IL-12. This cytokine has been identified as a key determinant in the generation of cell-mediated immunity (Trinchieri and Gerosa, *J. Leukocyte Biol.* **59**:505-511 (1996)). Animals treated with antibody to IL-12 fail to develop cell mediated immunity to intracellular pathogens (Id.). Conversely, susceptible animals treated with recombinant IL-12 develop resistance to infection with intracellular pathogens (Heinzel *et al.*, *J. Exp. Med.* **177**:1505-1512 (1993). IL-12 has been used as an adjuvant to specifically elicit cell-mediated immunity (Miller *et al.*, *J. Immunol.* **155**:4817-4828 (1995)).

D) Immunization Against *Rhodococcus equi* Infection

The use of antibiotics to prevent Rhodococcus infection is expensive and only partially effective. There are currently no vaccines against *R. equi* infection.

Immunization of experimental animals with the purified *R. equi* virulence-associated protein VapA does not reliably confer a high degree of protective immunity against R. equi pneumonia.

Effective prevention of *R. equi* pneumonia in foals by passive immunization using *R. equi* hyperimmune equine plasma has been reported (Martens *et al.*, *Equine Veterinary Journal* **21**:249-55 (1989)). Though this is the best prophylactic strategy available at present, it is impractical to implement on a large scale because of its inconvenience and extreme costs in terms of both product and manpower. It has also been reported that colostrum from immunized mares is not protective (Martens *et al.*, *Equine Veterinary Journal* **12**[Supp.]:19-26 (1991)).

There is a great need for improved methods and compositions for immunizing vertebrates, and in particular for immunizing foals, against Rhodococcus infection and against the pneumonia associated with *R. equi* infection.

E) Expression of Recombinant Nucleic Acids in Rhodococcus

Genetic systems for the transformation of Rhodococcus are still in the developmental phase. Several groups have transformed Rhodococcus and related nocardia. Desomer and colleagues used electroporation to transform *Rhodococcus fascians* with a fragment of an indigenous Rhodococcus plasmid conferring chloramphenicol resistance (Desomer *et al.*, *Mol. Micro.* **5**:2115-2124 (1991)). Vectors comprising indigenous Rhodococcus plasmid origins of replication are described in U.S. Patent Nos. 5,246,857 and 4,920,054. Quan and Dabbs recently transformed Rhodococcus with an arsenic resistance cloning vector (Quann and Dabbs, *Plasmid* **29**:74-79 (1993)). Finnerty and colleagues

have developed a *Rhodococcus-E. coli* shuttle vector which they have used to transform several nocardiaform species, including *R. equi* (Vogt Singer and Finnerty, *J. Bacteriol.* **170**:638 (1988), see also U.S. Patent No. 4,952,500).

The lack of plasmid stability has hampered these approaches, especially in *R. equi*, where plasmid stability has proven to be a considerable obstacle.

There is a need for improved methods and additional vectors for the recombinant expression of nucleic acids in Rhodococcus.

Summary of the Invention

The present invention is based upon the surprising discovery that an avirulent strain of *R. equi*, transformed with a gene encoding the virulence-associated VapA protein, is not virulent. The invention is further based upon the discovery that the transformed strain is a potent inducer of IL-12, an important component of cell mediated immunity, and that the transformed strain can induce protective immunity in animals.

The present invention therefore relates to the discovery of safe and effective methods and compositions for immunizing a vertebrate against Rhodococcus infection and against the pneumonia associated with *R. equi* infection.

The invention provides a method of immunizing a vertebrate against Rhodococcus comprising administering a vaccine to the vertebrate, wherein said vaccine comprises a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof. In a preferred embodiment the vertebrate is a mammal; in a more preferred embodiment the vertebrate is selected from the group consisting of pigs, horses, and humans; in a most preferred embodiment the vertebrate is a horse. Where the vertebrate is a horse the horse is preferably a foal.

In a preferred embodiment the nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof is DNA. In a more preferred

embodiment the DNA encoding VapA or an immunogenic fragment thereof is operatively linked to regulatory sequences. In a most preferred embodiment the vaccine further comprises a pharmaceutically acceptable carrier.

In a preferred embodiment the vaccine comprises recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof. The DNA encoding VapA or an immunogenic fragment thereof may be extrachromosomal or may be integrated into the bacterial chromosome. In some embodiments the bacteria further express an immunostimulatory cytokine.

In a more preferred embodiment the recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof are an avirulent strain of Rhodococcus. In a most preferred embodiment the recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof are an avirulent strain of *R. equi*.

In another more preferred embodiment the recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof are Mycobacteria. In a most preferred embodiment the recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof are Bacillus Calmette-Guerin (BCG).

In another preferred embodiment the vaccine comprises a pharmaceutically acceptable carrier and DNA encoding VapA or an immunogenic fragment thereof.

In some embodiments the DNA vaccine is administered to the vertebrate by injection. In some embodiments the vaccine is administered to the vertebrate by a gene gun. In a preferred embodiment the vaccine is administered into muscle or skin; in a most preferred embodiment the vaccine is administered into muscle.

This invention further provides a composition comprising a pharmaceutically acceptable carrier and recombinant bacteria which express DNA encoding VapA or an immunogenic fragment thereof. The DNA encoding VapA or an immunogenic fragment thereof may be extrachromosomal or may be

integrated into the bacterial chromosome. In some embodiments the recombinant bacteria are an avirulent strain of Rhodococcus; in a most preferred embodiment the recombinant bacteria are an avirulent strain of *R. equi*. In some embodiments the recombinant bacteria are Mycobacteria; in a most preferred embodiment the recombinant bacteria are BCG. In some embodiments the recombinant bacteria also express an immunostimulatory cytokine.

The invention also provides a method for expressing a recombinant nucleic acid of interest in Rhodococcus cells comprising:

(1) providing a recombinant vector which comprises the nucleic acid of interest and a mycobacterial origin of replication;

(2) transforming the Rhodococcus cells with said recombinant vector; and

(3) culturing the transformed Rhodococcus cells under conditions wherein the nucleic acid of interest is expressed.

In a preferred embodiment the nucleic acid of interest is operatively linked to regulatory sequences. In some preferred embodiments the recombinant vector further comprises an *E. coli* origin of replication. In one preferred embodiment the recombinant nucleic acid encodes a polypeptide of interest; in a more preferred embodiment the polypeptide of interest is recovered from the transformed cells.

The invention also provides a method of inducing an immune response in a vertebrate comprising administering recombinant Rhodococcus bacteria which express DNA encoding an immunogen. The DNA encoding the immunogen may be extrachromosomal or may be integrated into the bacterial chromosome. In some embodiments the recombinant bacteria also express an immunostimulatory cytokine. In a preferred embodiment the recombinant bacteria are *R. equi*.

The invention encompasses a method of preventing Rhodococcus infection in a vertebrate comprising administering a vaccine to the vertebrate, wherein said vaccine comprises a nucleic acid encoding the *R. equi* VapA protein

or an immunogenic fragment thereof, as well as a method of preventing pneumonia associated with Rhodococcus infection in a vertebrate comprising administering a vaccine to the vertebrate, wherein said vaccine comprises a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof. In a preferred embodiment the vertebrate is a horse; in a most preferred embodiment the vertebrate is a foal.

The invention also encompasses the use of a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof for the preparation of a vaccine against Rhodococcus.

Other aspects and advantages of the present invention are described in the drawings and in the following detailed description of the preferred embodiments thereof.

Description of the Drawings

Figure 1 shows the growth of virulence-associated plasmid-positive (103+, closed circles) and plasmid-negative (103-, open circles) strains of *R. equi* in murine macrophages. Growth is expressed as the number of bacteria per 200 macrophages (1A) or the number of macrophages (out of 200) with 10 or more bacteria in them (1B).

Figure 2 shows the growth of virulence-associated plasmid-positive (103+, triangles) and plasmid-negative (103-, circles) strains of *R. equi* in mice.

Figure 3 shows flow cytometry profiles of VapA expression in *R. equi* 103^+ (3A), 103^- pYUB415 (3B), and 103^- pYUB415-VapA (3C).

Figure 4 shows the growth of *R. equi* in the macrophage-like cell line J774. Growth is expressed as the number of bacteria per 200 macrophages (4A) or the number of macrophages (out of 200) with ten or more bacteria in them (4B).

Figure 5 shows the growth of 103^+ (squares), 103-415 (vector alone, triangles), and 103-VapA2 (103^- transformed with pYUB415-VapA, circles) *R. equi* in mice.

Figure 6 shows RT-PCR detection of IL-12 and iNOS mRNA.

Figure 7 shows the growth of *R. equi* strains in the liver (7A) and spleen (7B) of vaccinated mice.

Detailed Description of the Invention

This invention is based upon the surprising discovery that avirulent *R. equi* bacteria transformed with a gene (*vapA*) expressing VapA protein are not virulent, and that avirulent strains of *R. equi* are potent inducers of IL-12 by macrophages.

The present invention provides methods and compositions for immunizing a vertebrate against Rhodococcus infection, and for preventing pneumonia associated with *R. equi* infection. The invention further relates to methods for expressing recombinant nucleic acids in Rhodococcus cells.

A) Definitions

The following definitions, of terms used throughout the specification, are intended as an aid to understanding the scope and practice of the present invention.

"**Expression**" means the realization of genetic information encoded by a nucleic acid to produce a functional RNA or protein.

"**Foal**" means an equine animal less than one year in age.

"**Immune response**" means a cytotoxic T cell response or increased serum levels of antibodies to an antigen, or to the presence of neutralizing antibodies to an antigen such as a Rhodococcus protein. Immune response includes "**protective immunity**," which is the ability of the serum antibodies and/or cytotoxic T cell response to totally or partially protect against disease caused by an infectious agent such as Rhodococcus.

"**Immunize**," "**immunizing**," and "**immunization**" refer to the act of inducing an immune response.

"**Immunogenic fragment**" means a fragment of a polypeptide which is capable of inducing an immune response.

"**Operatively linked to regulatory sequences**" means that the polypeptide coding region is connected to transcriptional and translational regulatory sequences in such a way as to permit polypeptide expression when the appropriate molecules (such as activator proteins and polymerases) are present in a cell or cell free system.

"**Vaccine**" means any composition which is administered in order to induce an immune response.

B) Immunization

Immunological studies by the inventor and by others have highlighted the importance of cell mediated immunity (CMI) in host defense to intracellular pathogens such as *R. equi*. Humoral immunity can be difficult to induce early in development and it is often ineffective against intracellular pathogens, such as *R. equi*. The present invention encompasses methods and compositions to preferentially elicit cell mediated immunity. Vaccines according to the present invention comprise a nucleic acid encoding VapA or an immunogenic fragment thereof. One embodiment of the present invention is directed to the use of a vaccine comprising recombinant bacteria expressing VapA; another embodiment is directed to the use of a vaccine comprising naked DNA.

The vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof (including recombinant bacteria embodiments and naked DNA embodiments) may be administered to various tissues and cells of the animal body, including muscle, skin, brain, lung, liver, spleen, and blood. Routes of administration include but are not limited to intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterial, intraocular, and oral, as well as transdermal, and by inhalation or suppository. Administration of the vaccine is preferably by means of parenteral administration

to the cells of muscle or skin. The vaccine may be injected into muscle or skin using an injection syringe or a needleless injection device. The vaccine may also be delivered into muscle or skin using a gene gun.

In some embodiments, immunization comprises a single administration of the vaccine. In other embodiments the vaccine is administered at least twice over a period of time. The period of time between immunizations may include from 24 hours apart to two weeks or longer. Alternatively, at least two and up to four or more separate immunizations are given simultaneously at different sites.

The vaccine comprising the nucleic acid encoding VapA or an immunogenic fragment thereof includes a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, isotonic saline (*e.g.*, monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, or mixtures of such salts), Ringer's solution, dextrose, mannitol, sorbitol, lactose, water, sterile water, glycerol, ethanol, and combinations thereof. The compositions are preferably sterile and pyrogen free. In some embodiments stabilizers, such as gelatin or albumin, may be included in the composition.

The vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof may optionally be formulated with one or more response enhancing agents such as: compounds which enhance transfection, compounds which stimulate cell division, compounds which stimulate immune cell migration to the site of administration (inflammatory agents), compounds which enhance an immune response (adjuvants), or compounds having two or more of these activities.

Response enhancing agents include but are not limited to lectins, growth factors, cytokines and lymphokines such as α-interferon, γ-interferon, platelet derived growth factor (PDGF), G-CSF, GM-CSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-8, IL-10 and IL-12 as well as collagenase, fibroblast growth factor, estrogen, dexamethasone, saponins, surface

active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene and squalane, hyaluronic acid and hyaluronidase.

The vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof may also include or express additional antigens against which protective immunity is desired (multivalent vaccines).

The vaccine may be administered to foals or to newborn horses on farms with endemic *R. equi*. The vaccine may also be administered to foals or to newborn horses on farms without endemic *R. equi*, *i.e.*, prophylactically.

C) Vaccines Comprising Recombinant Bacteria Expressing VapA

One preferred embodiment of the present invention is a vaccine comprising recombinant bacteria expressing VapA or an immunogenic fragment thereof. The VapA may be expressed from the *vapA* gene which is either extrachromosomal or integrated into the bacterial chromosome.

The vaccine compositions according to this embodiment contain about 1×10^5 to about 1×10^{10} bacteria. In a preferred embodiment about 1×10^6 to about 1×10^8 bacteria are administered in each immunization. In a more preferred embodiment about 5×10^6 to about 5×10^7 bacteria are administered, and in a most preferred embodiment about 1×10^7 bacteria are administered in each immunization.

In a preferred embodiment the recombinant bacteria are an avirulent strain of *Rhodococcus equi*. The inventor has shown that avirulent strains of *R. equi* are potent inducers of IL-12 from macrophages. IL-12 stimulates cell-mediated immunity, the component of the immune system most important for *R. equi* clearance, and the part that is most affected in AIDS patients. *R. equi* is therefore expected to be an excellent vaccine vector, especially where it is important to stimulate cell mediated immunity.

In another preferred embodiment the recombinant bacteria are the relatively avirulent mycobacterium called BCG (Bacillus Calmette-Guerin). Because BCG is antigenically similar to *Mycobacterium tuberculosis*, it is routinely used to vaccinate humans in Eastern European and Asian countries against tuberculosis. In fact, BCG is one of the most widely used human vaccines. In the past 35 years it has been administered to over 2.5 billion people, with remarkably few adverse effects. Thus, BCG is considered to be "safe" as a vaccine vector. BCG can be given at birth and engenders long-lived immune responses after a single immunization. Recombinant BCG can be used to induce both humoral and cellular immunity.

In BCG, the VapA or immunogenic fragment thereof may be expressed from a stably integrated copy of all or part of the *vapA* gene or from an extrachromosomal copy of all or part of the *vapA* gene, using the methods described in U.S. Patent Nos. 5,504,005 and 5,591,632 which are incorporated herein by reference. In some embodiments, the BCG also express a secreted immunostimulatory cytokine which is believed to enhance the immune response. Immunostimulatory cytokines which may be expressed include but are not limited to interferons (including α, β, and γ-interferon), the interleukins (including interleukins 1-12), tumor necrosis factors (including TNF α and β), and the colony stimulating factors (including M-CSF, G-CSF, and GM-CSF).

D) Vaccines Comprising Naked DNA

Another preferred embodiment of the present invention is a vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof. The nucleic acid can be DNA or RNA. In a preferred embodiment the nucleic acid is naked DNA.

The theory behind this vaccination approach is that the DNA is delivered into mammalian cells where it is transcribed and translated into the VapA protein. This expressed protein then serves as the immunogen to elicit an

immune response to *R. equi*. The immune response elicited by DNA vaccines is broad-based, including both humoral and cell mediated responses.

In a more preferred embodiment the DNA comprises a cDNA encoding the entire VapA protein. In other more preferred embodiments the DNA encodes an immunogenic fragment of VapA. DNAs encoding fragments of VapA can be constructed using the techniques of recombinant DNA technology, which are known to those of ordinary skill in the art. General methods for the cloning and expression of recombinant molecules are described in Sambrook *et al.* Molecular Cloning, a Laboratory Manual 2d Ed., Cold Spring Harbor Laboratories 1989), and in Ausubel (Current Protocols in Molecular Biology, Wiley and Sons, 1987), which are incorporated herein by reference. The immunogenicity of a fragment can be tested using standard immunological methods and the techniques disclosed herein.

The DNA encoding VapA or an immunogenic fragment thereof may be operatively linked to regulatory sequences which include promoters and polyadenylation signals. Other regulatory sequences include translation initiation signals (Kozak regions), termination codons, and enhancers. The regulatory sequences used must be functional within the cells of the vertebrate to be immunized.

Examples of promoters that can used in the present invention include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine and human metallothionein. In a preferred embodiment the promoter is the CMV immediate early promoter, as described in U.S. Patent Nos. 5,168,062 and 5,385,839 which are incorporated herein by reference.

Examples of polyadenylation signals that can be used in the present invention include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

In order to be a functional genetic construct, the regulatory sequences must be operably linked to the nucleic acid that encodes VapA. Accordingly, it is necessary for the initiation and termination codons to be in frame with the coding sequence.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the immunized vertebrate. Moreover, codons may be selected which are most efficiently transcribed in the immunized vertebrate. One having ordinary skill in the art can produce DNA constructs which are functional in the cells of the vertebrate to be immunized.

In some embodiments the vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof comprises a plasmid expression vector. In other embodiments the vaccine comprising a nucleic acid encoding VapA or an immunogenic fragment thereof comprises a self-amplifying replicon such as an alphaviral replicon (Berglund *et al.*, *Nature Biotechnology* 16:562-65 (1998)).

Enhancing agents can be used to improve the efficiency of nucleic acid vaccines.

In one embodiment, Cardiotoxin is injected before the injection of the nucleic acid. This procedure has been shown to enhance the efficiency of gene transfer (Whalen *et al.*, *Dev. Biol.* **141**:24-40 (1990)).

In another embodiment, bupivacaine, or a related response enhancing agent, is administered prior to, simultaneously with or subsequent to the nucleic acid, as described in U.S. Patent No. 5,593,972 which is incorporated herein by reference. Bupivacaine promotes and facilitates the uptake of genetic material by the cell, so administration of the nucleic acid in conjunction with bupivacaine facilitates entry of the genetic constructs into cells. Bupivacaine is believed to disrupt or otherwise render the cell membrane more permeable, and to stimulate cell division. Administration of bupivacaine also irritates and damages the tissue. As such, it acts as an inflammatory agent which elicits migration and chemotaxis of immune cells to the site of administration.

The vaccine compositions according to this embodiment comprise about 1 nanogram to about 1000 micrograms of DNA. In some preferred embodiments, the vaccines comprise about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the vaccines comprise about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the vaccines comprise about 1 to about 350 micrograms of DNA. In some preferred embodiments, the vaccines comprise about 25 to about 250 micrograms of DNA. In some preferred embodiments, the vaccines comprise about 100 micrograms DNA.

E) Immunological Methods

The skilled artisan will adjust the dosage, formulation, and schedule and route of administration to suit the specific circumstances and needs of the vertebrate being immunized. The skilled artisan will make use of several well characterized immunological methods for the routine optimization of the methods and compositions according to the present invention.

The expression of VapA, cytokines, or other proteins or peptides in a sample may be determined by contacting the sample with a polyclonal or monoclonal antibody which recognizes the molecule of interest. Methods for measuring protein expression include the "Western" blotting technique,

fluorescence activated cell sorting (FACS), and enzyme-linked immunosorbent assays (ELISA).

Polyclonal antibodies may be prepared by administering VapA, or a fragment, derivative, or epitope thereof, to an animal then harvesting antisera using techniques and procedures known in the art.

Monoclonal antibodies may be prepared using methods which are well known to those of skill in the art. Briefly, VapA protein is used to immunize spleen cells of Balb/C mice. The immunized spleen cells are fused with myeloma cells. Fused cells containing spleen and myeloma cell characteristics are isolated by growth in HAT medium, a medium which kills both parental cells, but allows the fused products to survive and grow. The preparation of monoclonal antibodies specific for VapA (designated Mab103) has been described (Tan *et al.*, *Can. J. Vet. Res.* **59**:51-59 (1995)).

The expression of mRNAs encoding cytokines or other gene products may be determined using the "Northern" blotting technique, RT-PCR (reverse transcription followed by polymerase chain reaction), or other methods known to those of skill in the art.

The course of infection with *R. equi*, as well as the optimization of immunological protocols, is advantageously studied using mice as an animal model. The course of infection may be followed by measuring the number of bacteria in the liver, lung, and spleen over time, using a colony forming assay.

Immunological parameters can also be examined in vaccinated mice, and these can be compared to control mice. Serum can be drawn from immunized animals to measure antibodies to Rhodococcus or to VapA. T cells are isolated from both the spleens and the lymph nodes of mice and exposed to either crude *R. equi* supernatants as a source of antigen, or to partially purified VapA. T cell proliferation is measured by the incorporation of tritiated thymidine. The production of IL-4 and γ-interferon by T cells from vaccinated mice is determined by RT-PCR (mRNA) and by ELISA (protein). To analyze macrophage activation, macrophages are washed from the peritoneal cavity of

mice at varying times post-vaccination. Biochemical and functional studies are performed on these cells. The transcription of the gene for the inducible form of nitric oxide synthetase (iNOS) is the best marker for macrophage activation in the murine system. Transcription of iNOS is analyzed using RT-PCR. The production of nitric oxide is measured by the Griess reagent. Functional assays can also be performed on these macrophages. The inventor has shown that virulent isolates of *R. equi* cannot survive in macrophages that have been activated *in vitro* by exposure to γ-interferon. Macrophages from vaccinated mice are incubated with *R. equi* and the number of bacteria surviving after 48 hours is determined, and compared to macrophages from nonvaccinated mice. These molecular and functional criteria are used to determine the activation state of macrophages in vaccinated animals. These studies allow the optimization of the efficacy of each of the vaccine approaches.

The level of protective immunity is advantageously quantitated by measuring clearance from the lung. Virulent *R. equi* are delivered via an aerosol route in order to mimic the natural route of infection. To aerosolize bacteria into the lungs of mice, the mice are placed into a sealed container within a standard aerosol generator machine equipped with a venturi unit. This unit produces an aerosol mist of bacteria that the mice inhale. For the administration of virulent organisms, the suite in which the generator is placed is under negative pressure with respect to atmospheric pressure and all air leaving the room is HEPA filtered.

For horses, the aerosol is generated by an ultrasonic nebulizer with a disposable drug vial (Hospital Medical Co. Montreal, Quebec, CA). Approximately 20 ml of solution containing from 1 x 10^7 to 1 x 10^9 bacteria/ml are placed into the vial which is attached to an inhalation mask. Foals are challenged for approximately 15 min with 10-20 ml of the suspension (Chirino-Treho *et al.*, *Can. J. Vet. Research* **51**:444-47 (1987).

To optimize the methods and compositions according to the invention, animals are first immunized with a VapA vaccine and then increasing

does of virulent bacteria are aerosolized into the lungs. The number of bacteria in the lung, liver, and spleen is quantitated over time.

F) Rhodococcus Expression Systems

In one embodiment of the present invention, a recombinant nucleic acid of interest is expressed in Rhodococcus cells. The Rhodococcus cells are transformed with a vector which comprises the nucleic acid of interest and also a mycobacterial origin of replication. In a preferred embodiment the vector further comprises a second origin of replication, which allows the vector to replicate in a microorganism such as *E. coli*, *B. subtilis*, or *S. aureus*. These vectors are commonly called "shuttle vectors". The vector preferably comprises one or more selectable markers such as antibiotic or heavy metal resistance.

In one preferred embodiment, the Rhodococcus expression system is used to produce a polypeptide of interest. Rhodococcus cells are transformed with a recombinant vector which expresses the polypeptide of interest. The Rhodococcus cells are then cultured under conditions that allow the polypeptide to be produced, and the polypeptide is recovered form the cultured cells.

In another preferred embodiment the Rhodococcus expression system is used as a vaccine. A nucleic acid encoding an antigen is transformed into the Rhodococcus expression system, and the transformed bacteria are used to immunize a vertebrate and thus elicit protective immunity against the antigen. The ability of Rhodococcus to stimulate the production of IL-12 by macrophages makes it a good candidate for the delivery of antigens when cell mediated immunity is desired. Examples of this approach which may be mentioned include the expression of *M. tuberculosis* antigens, *M. laprae* antigens, and HIV antigens in Rhodococcus, and the use of the transformed bacteria to immunize a vertebrate against tuberculosis, leprosy, or AIDS.

Examples

The following examples illustrate the invention. These examples are illustrative only, and do not limit the scope of the invention.

EXAMPLE 1

Selection of an Avirulent Strain of *R. equi*

Avirulent organisms, lacking the large virulence-associated plasmid, were developed by repeated passage of an originally virulent strain in culture. During passage in culture some of the organisms lose their plasmids. By repeated passage, a population is obtained in which a significant number of bacteria lack plasmids.

A plasmid-positive organism (designed herein as "103+" or "103^+") was passaged for 50 generations in culture. The population was then plated and several colonies were picked and analyzed. A plasmid-negative isolate (designated herein as "103-" or "103^-") was selected.

EXAMPLE 2

The Growth of Virulent and Avirulent Strains of *R. equi* in Macrophages

The intracellular growth in macrophages of a strain of *R. equi* containing the large virulence-associated plasmid (103+) was compared to the growth of the isogenic strain lacking this plasmid (103-).

Intracellular growth of *R. equi* in macrophages was measured using an immunofluorescence-based assay (Hondalus and Mosser, *Infect. and Immun.* **62**:4167-4175 (1994)). Briefly, parallel monolayers were differentially fixed with either methanol or paraformaldehyde. Methanol fixation permeabilizes the macrophage cell membrane so that both extracellular and intracellular bacteria are visible following staining, whereas paraformaldehyde fixation permits quantitation of extracellular bacteria only.

Plasmid-positive or plasmid-negative bacteria were added to monolayers of murine macrophages for one hour. After washing, intracellular

growth was measured over the next 48 hours. For each sample, 200 macrophages were counted. Bacterial growth was expressed as either the number of bacteria per 200 macrophages or the number of macrophages (out of the 200 counted) with ten or more bacteria.

As shown in Figure 1, the strain containing the virulence-associated plasmid (103+) grew efficiently in macrophages. By 48 hours postinfection, there was an increase in the total number of intracellular bacteria associated with the monolayers and most of the infected cells contained 10 or more bacteria. In contrast to the plasmid-positive strain, the plasmid-negative strain (103-) was not able to replicate efficiently in macrophages. The two strains of bacteria exhibited similar extracellular growth kinetics in broth and on plates.

EXAMPLE 3

The Growth of Virulent and Avirulent Strains of *R. equi* in Macrophages and Mice

Mice are the species of choice to measure virulence of *R. equi* (Takai *et al.*, *J. Clin. Micro.* **29**:439-43 (1991)). The virulence of a strain of *R. equi* containing the large virulence-associated plasmid was compared to the virulence of a strain lacking this plasmid.

Groups of mice were injected intravenously with equal numbers of plasmid-positive or plasmid-negative bacteria. At five days post-infection the livers and spleens were removed and the number of bacteria in each organ was quantitated by plating serial dilutions of whole organ homogenates.

As shown in Figure 2, by day five post-infection the number of plasmid-negative bacteria decreased to undetectable levels. In contrast, the organs of all animals infected with the plasmid-positive strain contained numerous viable bacteria.

EXAMPLE 4

The Expression of VapA in Avirulent Bacteria

The results from Examples 2 and 3 showed that presence of the large plasmid correlates with increased intracellular growth in macrophages, and with increased bacterial burdens in the liver and spleens of infected mice (*in vivo*). Bacteria lacking the plasmid were avirulent in experimentally infected mice. These avirulent organisms were next transformed with the VapA gene derived from the virulence-associated plasmid.

A) Construction of a Shuttle Vector

The pYUB415 vector was provided by Dr. William Jacobs (Albert Einstein College of Medicine, Bronx, N.Y.). The pYUB415 plasmid is a 9301 bp mycobacterium-*E. coli* shuttle vector. It contains the colE1 origin of replication for *E. coli* and the pAL5000 origin of replication for mycobacteria. It bears a *bal* gene encoding ampicillin resistance in *E. coli* and the *hyg* gene encoding hygromycin resistance in mycobacteria.

A pBluescript (Stratagene, LaJolla, CA) plasmid containing the *vapA* gene, from the large "virulence-associated" 85 kbp plasmid of wild-type *R. equi* strain 103+, was provided by Dr. J. Prescott (University of Guelph, Ontario, Canada, *see Can. J. Vet. Res.* **59**:51-59 (1995)). A 1.6 kbp BamHI/EcoRV fragment encoding VapA was subcloned into BamHI/EcoRV-digested pYUB415. A resulting clone, designated "pYUB415-vapA," was transformed into *E.coli* and *R. equi*.

B) Transformation of an Avirulent Strain

The pYUB415-VapA plasmid was transformed into the 103- isolate (See Example 1) of *R. equi*. Electroporation was performed using a Gene Pulser (BioRad, Melville, NY) set at 1.25 kV, 25 uF, and 400 ohm, in the presence of approximately 1 μg DNA in a 0.1 cm electroporation cuvette. As a control, the 103- strain was transformed with the pYUB415 vector alone (103$^-$ pYUB415).

Transformed bacteria were selected on hygromycin and analyzed for VapA expression.

C) VapA Expression in Transformed Bacteria

Western blot analysis showed that the transformed bacteria express the VapA protein.

Flow cytometry showed that the transformed bacteria express the VapA protein on their surface. Bacteria were stained by indirect immunofluorescence using culture supernatants containing a monoclonal antibody (mAb 103) to VapA. As shown in Figure 3, the expression of VapA on the transformants (103^- pYUB415-VapA) was comparable to wild-type (103+) organisms. VapA expression on transformed bacteria was stable in culture in the presence of antibiotics, as shown in Table 1.

Table 1 shows a flow cytometry analysis on bacteria from five successive passages. There was no loss in VapA levels on the surface of transformed bacteria. VapA expression on transformed bacteria was temperature sensitive, similar to that on wildtype bacteria.

Table 1

Strain		% Positive	MFI*
103+ (irrelevant antibody)		1	0.285
103- pYUB415		41.8	0.756
103+	Passage 1	82.2	5.71
103+	Passage 2	86.1	7.77
103+	Passage 3	86.6	8.66
103+	Passage 4	87.7	9.21
103+	Passage 5	86.6	10.2
103- VapA	Passage 1	78.1	4.61
103- VapA	Passage 2	82.4	6.47
103- VapA	Passage 3	83.2	7.01
103- VapA	Passage 4	89.3	8.03
103- VapA	Passage 5	80.9	5.27

*Mean Fluorescence Intensity

The following experiment showed that VapA expression in the transformed bacteria is stable even in the absence of antibiotic selection. *R. equi* strains 103+ (bacteria containing the large virulence associated plasmid), 103-415 (103- plasmid-cured strain which has been transformed with the pYUB415 vector alone), and 103-VapA2 and 103-VapA3 (two 103- transformants containing the pYUB415-VapA plasmid) were cultured in the absence or presence of 150 μg/ml hygromycin. The bacteria were passaged once each day. The expression of VapA was monitored by flow cytometry using an anti-VapA primary antibody and a goat anti-mouse FITC secondary antibody. As shown in Table 2, the VapA plasmid was stably expressed over seven passages even in the absence of hygromycin. These data indicate that vaccines according to the

present invention can be used to express the VapA antigen in a vertebrate without the use of antibiotic selection.

Table 2

Strain	Passage	Hygromycin	% Positive	MFI
103+ control	O/N	absent	1.1	1.27
103+	O/N	absent	90.9	9.37
103+	1	absent	81.7	8.10
103+	2	absent	86.4	6.80
103+	3	absent	82.0	8.20
103+	4	absent	80.6	8.52
103+	5	absent	69.3	9.71
103+	6	absent	55.5	9.36
103+	7	absent	39.0	8.0
103-415	O/N	present	0.3	1.80
103-415	1	present	2.2	1.45
103-415	2	present	0.7	2.22
103-415	3	present	1.5	1.34
103-415	4	present	1.9	1.32
103-415	5	present	1.5	1.30
103-415	6	present	1.4	1.33
103-415	7	present	1.4	1.42

Strain	Passage	Hygromycin	% Positive	MFI
103-VapA3	O/N	present	86.5	6.31
103-VapA3	1	present	80.0	5.51
103-VapA3	2	present	82.2	5.18
103-VapA3	3	present	83.8	5.49
103-VapA3	4	present	83.8	5.61
103-VapA3	5	present	82.7	5.27
103-VapA3	6	present	84.2	5.31
103-VapA3	7	present	83.3	4.81
103-VapA3	1	absent	79.8	5.24
103-VapA3	2	absent	79.1	5.41
103-VapA3	3	absent	81.6	5.51
103-VapA3	4	absent	79.9	6.21
103-VapA3	5	absent	80.8	5.89
103-VapA3	6	absent	79.6	5.84
103-VapA3	7	absent	81.5	5.51
103-VapA2	O/N	present	86.1	7.14
103-VapA2	1	present	79.0	7.32
103-VapA2	2	present	87.7	6.87
103-VapA2	3	present	81.6	6.92
103-VapA2	4	present	82.0	7.04
103-VapA2	5	present	84.5	7.34
103-VapA2	6	present	86.4	6.76
103-VapA2	7	present	89.5	7.48

Strain	Passage	Hygromycin	% Positive	MFI
103-VapA2	1	absent	82.2	6.18
103-VapA2	2	absent	87.2	6.94
103-VapA2	3	absent	78.5	6.75
103-VapA2	4	absent	85.6	5.94
103-VapA2	5	absent	82.8	6.92
103-VapA2	6	absent	81.7	7.42
103-VapA2	7	absent	83.8	7.35

O/N is overnight

MFI is mean Fluorescence Intensity

"103+ control" is 103+ stained with an irrelevant IgG1 isotype control antibody (Mopc21)

D) Growth of Transformed Bacteria in Macrophages and in Mice

Surprisingly, the bacteria transformed with *vapA* were avirulent and did not grow in macrophages under conditions where the virulent strains exhibited good intracellular replication. These bacteria also failed to grow efficiently in the macrophage-like cell line J-774. This cell line has reduced microbicidal activity relative to primary macrophages, and these cells are generally very permissive to intracellular growth.

Bacteria were added to J-774 cell monolayers for one hour, washed, and then examined over the next 48 hours by fluorescence microscopy. The average number of bacteria/macrophage and the number of macrophages (out of 200) with ten or more bacteria were quantitated. The *vapA*-transformed bacteria failed to grow efficiently in J-774 cells, as shown in Figure 4. Over the 48 hour assay period, the number of wildtype bacteria (103+) grew intracellularly in macrophages, as expected. Both the number of bacteria/macrophage and the number of macrophages with ten or more bacteria showed significant increases. The avirulent parent isolate did not demonstrate intracellular growth, as expected. The VapA-expressing transformant (103-

VapA2) behaved similarly to the avirulent isolate. These organisms exhibited little intracellular growth at 48 hours and the number of macrophages with ten or more bacteria increased only slightly. Thus, the 103-VapA isolates are not able to efficiently replicate in macrophages or macrophage-like cell lines.

To examine growth of the transformed bacteria in mice, mice were infected with these organisms and bacterial burdens in the livers and spleens were measured over the next week. Groups of five mice each were injected with 2-5 x 10^6 virulent 103+ bacteria or avirulent 103- bacteria that were transformed with either the 415 vector alone or with the 415 vector including the *vapA* gene. Spleen and liver burdens were determined at five days postinfection by a colony forming unit assay.

As shown in Figure 5, the transformed organisms (bacteria transformed with vector alone or vector expressing VapA) were completely cleared from infected mice. By five days post-infection there were no viable bacteria in either the liver or spleen. At this time the virulent organisms were exhibiting peak growth. Thus, the *vapA* gene product alone is not sufficient to confer virulence to *R. equi*.

EXAMPLE 5

IL-12 Production by Infected Macrophages

A semi-quantitative competitive reverse-transcriptase polymerase chain reaction (RT-PCR) was used to measure the expression of IL-12 and the inducible form of nitric oxide synthetase (iNOS). IL-12 is an effective stimulator of cell-mediated immunity (CMI), which is the component of the immune system most important for the clearance of intracellular pathogens. Part of the mechanism of action of IL-12 is to induce NK cells to produce γ-interferon, a molecule which activates macrophages to kill intracellular pathogens such as *R. equi*. The transcription of iNOS is a marker for macrophage activation in the murine system.

Monolayers of resident and γ-interferon primed macrophages (approx. 1 x 10^5 cells/monolayer) were incubated with increasing amounts of *R. equi*, ranging from 2 x 10^5 to 2.5 x 10^6 bacteria/monolayer (a ratio of 2-25 bacteria/macrophage) in the presence of serum as an opsonin to promote bacterial uptake into the macrophages. A virulent clinical isolate, 238, was used in the initial studies. After 30 minutes of incubation, the cells were washed and then incubated for an additional 6 or 24 hours. Cells were lysed in RNAzol B (Tel-Test, Inc. Friendswood, TX) according to the manufacturer's instructions, and total mRNA was obtained. The RNA was reverse transcribed and the cDNA was amplified by the polymerase chain reaction along with a competitive mimic. The pPQRS plasmid contains a number of competitive mimic cytokines, and was provided by Dr. Steven Reiner (University of Chicago Medical School) (Reiner *et al.*, *J. Immunol. Methods.* **165**:37-46 (1993)). A known concentration of the pPQRS plasmid was added to each reaction. The results are shown in Figure 6. The upper band on the gels is the competitive mimic and the lower band is the actual cDNA. The amount of cDNA was deduced by comparing the intensities of the upper and lower bands. Input cDNA was normalized to the housekeeping gene, HPRT.

As shown in Figure 6, macrophages exposed to *R. equi* alone or *R. equi* plus γ-interferon made detectable amounts of IL-12. The 238 isolate induced the production of low levels of IL-12 in resting macrophages and higher levels of IL-12 in interferon-primed macrophages. *R. equi* is therefore a potent stimulator of IL-12 even by resident (non-activated) macrophages. Similar results were obtained using avirulent (virulence associated plasmid-negative) bacteria.

EXAMPLE 6

Transformed Avirulent Bacteria as a Vaccine

The unexpected finding that the avirulent strain transformed with a gene expressing VapA is not virulent (Example 4), combined with the discovery that avirulent strains of *R. equi* are potent inducers of IL-12 by macrophages

(Example 5), suggested that the transformed *R. equi* would be an effective vaccine.

A) Immunization of Mice

Five mice per group were vaccinated with 2 x 10^6 viable wildtype *R. equi* (103+), strain 103- transformed with vector alone (103 - 415), or strain 103- transformed with a plasmid encoding VapA (103 - VapA). After two weeks, mice were infected intravenously with 1 x 10^7 virulent 103+ bacteria. After five days the liver (Fig. 7A) and spleens (Fig. 7B) were removed and the number of viable bacteria was determined by dilution plating.

As shown in Figure 7, mice vaccinated with avirulent bacteria had relatively high numbers of bacteria in their livers and spleens. The lack of protection by the avirulent strain may be because it is either cleared too rapidly to elicit an immune response or because it lacks the appropriate stimulatory antigens, or both. Mice infected with the same number of wildtype *R. equi* (103+) cleared the majority of their bacteria, showing that mice which recover from infection with wildtype bacteria are protected from subsequent infection.

Mice vaccinated with transformed attenuated *R. equi* expressing recombinant VapA (103-/VapA) exhibited significant reductions in the number of bacteria in their livers and spleens, relative to mice vaccinated with avirulent bacteria. Mice vaccinated with avirulent bacteria transformed with vector alone contained an average of 98,480 bacteria/liver $\pm$ 27,279 (SD). Mice vaccinated with avirulent bacteria expressing recombinant VapA contained an average of 16,010 bacteria/liver $\pm$ 813 (SD). This represents an 84% reduction in the number of bacteria in the liver. Because the positive control for bacterial growth was measured in mice that were vaccinated with avirulent bacteria (rather than unvaccinated mice), the protection observed in Figure 7 may actually be an underestimation relative to what might occur in a naive animal that has never been exposed to Rhodococcus.

B) Immunization of Horses

To confirm that the avirulent bacteria expressing VapA are avirulent in horses, bacteria (*R. equi* strains 103+, 103-, and 103-VapA) were aerosolized into the lungs of foals.

Eight three-week old foals were infected intrabronchially with a standardized dose (approx. 1 x 10^9 bacteria) of a virulent strain of *R. equi* containing the large virulence associated plasmid and expressing VapA (103+), eight foals were infected with the plasmid cured derivative of strain 103 (103-), and six foals received only saline (controls). In each group, half of the foals were euthanized three days post infection and half of the foals were euthanized fourteen days post infection. In addition, three foals were infected with avirulent 103- bacteria transformed with the *Mycobacterium-E. coli* 415 shuttle vector encoding VapA (103-VapA) and euthanized fourteen days post infection. The 103+ and 103-VapA strains expressed VapA to the same extent *in vitro*.

After infection the heart rate, respiratory rate, temperature, white blood cell count and fibrinogen concentration were monitored in the foals. After euthanasia the foals were subjected to a post-mortem examination.

The foals infected with the 103+ strain developed significantly higher temperatures, heart rates, respiratory rates, and fibrinogen concentrations than the control groups begining nine days post infection. The foals infected with 103- and 103-VapA remained asymptomatic.

On post-mortem examination, the foals administered 103-, 103-VapA, or saline showed no gross lung lesions. In contrast, all of the foals infected with 103+ had lesions ranging from mild to moderate consolidation of the cranio-ventral lung lobes on day 3, to a severe pyogranulomatous pneumonia involving more than 60% of the lungs on day 14. At three days post infection the mean number of *R. equi* bacteria in the lungs ($\log_{10}$ per gram of lung) was 3.67 $\pm$ 1.35 for foals infected with 103+, and 1.43 $\pm$ 0.73 for foals infected with 103- ($P = 0.05$). At fourteen days post infection the mean number of *R. equi* bacteria in the lungs was 9.45 $\pm$ 1.0 for foals infected with 103+, and 0.11 $\pm$

0.16 for foals infected with 103-VapA. *R. equi* could not be cultured at day 14 from the foals infected with 103-. *R. equi* was not cultured from the controls. These results illustrate the safety of the Rhodococcus vaccines according to the present invention.

To further show the efficacy of the vaccine according to the present invention, foals are infected with *R. equi* strains 103+, 103-, and 103-VapA as above, but the foals are allowed to mature rather than being euthanized after infection. At various times after infection antibody levels in the serum (including antibodies reactive to *R. equi* generally and to VapA specifically) are determined.

EXAMPLE 7

Avirulent Bacteria Having an Integrated VapA Gene

To avoid the need for antibiotic pressure and to avoid introducing antibiotic resistance genes into the environment, avirulent *R. equi* which express VapA but do not contain antibiotic resistance markers are constructed. These organisms express VapA from a *vapA* gene which is integrated into the bacterial chromosome.

The *vapA* gene is subcloned into a "suicide vector" containing a kanamycin resistance gene (*aph*) flanked by res- recombinase binding sites. The suicide vector can replicate in *E. coli* but not *R. equi*. Thus transformation of *R. equi* and selection for kanamycin resistance identifies those bacteria in which the plasmid has randomly and illegitimately integrated into the chromosome. Since chromosomal location can affect gene expression, several clones are screened for VapA expression. In those clones stably expressing levels of the VapA protein approaching that of wildtype, the kanamycin resistance gene is removed. Removal of the antibiotic resistance marker is accomplished by transforming these clones with a replicating plasmid containing a hygromycin resistance gene and expressing the gamma-delta-resolvase. The resolvase binds to the res sequences and through site-specific recombination, the intervening kanamycin resistance

gene is cleaved out. Kanamycin sensitive clones are passaged in the absence of antibiotic to encourage loss of the replicating plasmid. Recombinants that express VapA and are kanamycin and hygromycin sensitive are used to immunize foals as described in Example 6.

EXAMPLE 8

Naked DNA as a Vaccine

A) Expression Construct

Any mammalian expression vector having a strong eukaryotic promoter, such as the CMV promoter, can be used to express the *vapA* gene in mammalian tissues. Examples of expression vectors which can be used include CDM8 and Ap^{r}M8, each of which comprises a CMV promoter and a multicloning site into which genes can be inserted (Moser *et al.*, *J. Cell Biology* **116**:511-20 (1992); Rosenthal *et al.*, *Infection and Immunity* **64**:2206-15 (1996)).

The CDM8 and Ap^{r}M8 plasmids were provided by Dr. Tim Springer (Harvard Medical School) and Dr. Lloyd Klickstein (formerly of Johns Hopkins Medical School) respectively. A DNA fragment containing the *vapA* gene is cloned into each plasmid. Recombinant plasmid DNA is isolated using standard methods.

B) Immunization of Mice

Mice are injected in the hind muscle with approximately 100 μg of plasmid DNA containing the *vapA* gene. The direct gene transfer protocol into the tibialis anterior (TA) muscle of mice has been previously published (Davis *et al.*, *Human Molecular Genetics* **2**:1847-1851 (1993)). Briefly, 50 μg of purified plasmid DNA are injected into each of the two TA muscles, using a 27G needle. Mice are anesthetized, shaved and injected slowly with 50 μl of DNA in sterile endotoxin-free saline containing 20% sucrose. A small piece of polyethylene tubing is fit over the needle, allowing only 2.5 mm of needle to protrude, to optimize the depth of injection. DNA is injected into intact muscles. Animals

are examined 2-3 weeks later for antibody titres to *R. equi* and VapA. Some mice are treated with a single injection of Cardiotoxin (Sigma, St. Louis) seven days before the injection of DNA.

Three weeks after injection of DNA, mice are challenged with a dose of 1 x 10^7 cfu of virulent *R. equi*, a dose which is just below the LD50. One week after infection the animals are sacrificed and the livers, lungs, and spleens are harvested. The number of viable bacteria per organ is enumerated. Immunological studies are also performed.

C) Immunization of Horses

The DNA encoding VapA is administered to horses by injection or by use of the so-called "gene gun".

For injection, about 100 μg DNA is injected into the muscle using a needle.

The gene gun is an apparatus that injects small beads coated with DNA. Each firing of the gun injects about 0.5 mg of beads which contains approximately 0.5 μg DNA. For each immunization, the horses are anesthetized and "shot" approximately 25 times, to deliver approximately 15 μg DNA. Horses are immunized about two times.

EXAMPLE 9

Expression of VapA in Mycobacteria

The *vapA* gene is cloned into *E. coli*-mycobacterial shuttle vectors and into integrating vectors (Stover *et al.*, *Nature* **351**:456-59 (1991)).

The shuttle vector plasmid designated pMV261 was obtained from Dr. W. Jacobs and Dr. B. Bloom (Albert Einstein College of Medicine, Bronx, N.Y.). This is an extrachromosomal replicon containing an *E. coli* origin of replication (*oriE*) and the Tn903-derived *aph* gene conferring kanamycin resistance for use as a selectable marker for both *E. coli* and mycobacteria. The vector also contains an origin of replication for mycobacteria. In addition, the

vector contains an expression cassette consisting of the mycobacterial hsp60 promoter, a multiple cloning site and a transcription terminator.

The pMV361 vector (Dr. W. Jacobs) is an integrating vector in which the oriM of pMV261 has been removed and replaced with DNA carrying the attachment site (*attP*) and integrase (*int*) gene of mycobacteria phage L5. Thus, this vector can integrate into the mycobacterial chromosome via site-specific recombination.

Vectors containing the *vapA* gene are transformed into mycobacteria by electroporation. Expression of the VapA protein is demonstrated using Western blot techniques.

All references discussed herein are incorporated by reference, including United States Provisional Application Serial No. 60/053,937.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

CLAIMS

WE CLAIM

1. A method of immunizing a vertebrate against Rhodococcus comprising administering a vaccine to the vertebrate, wherein the vaccine comprises a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof.

2. The method of claim 1 wherein the nucleic acid is DNA.

3. The method of claim 2 wherein the DNA encoding VapA or an immunogenic fragment thereof is operatively linked to regulatory sequences.

4. The method of claim 3 wherein the vaccine comprises recombinant bacteria which express the DNA encoding VapA or an immunogenic fragment thereof.

5. The method of claim 4 wherein the DNA encoding VapA or an immunogenic fragment thereof is extrachromosomal.

6. The method of claim 4 wherein the DNA encoding VapA or an immunogenic fragment thereof is integrated into the bacterial genome.

7. The method of claim 4 wherein the bacteria are an avirulent strain of Rhodococcus.

8. The method of claim 7 wherein the bacteria are an avirulent strain of *Rhodococcus equi*.

9. The method of claim 8 wherein the DNA encoding VapA or an immunogenic fragment thereof is extrachromosomal.

10. The method of claim 8 wherein the DNA encoding VapA or an immunogenic fragment thereof is integrated into the bacterial genome.

11. The method of claim 4 wherein the bacteria are Mycobacteria.

12. The method of claim 11 wherein the bacteria are BCG.

13. The method of claim 4 wherein the bacteria also express an immunostimulatory cytokine.

14. The method of claim 3 wherein the vaccine is a composition comprising a pharmaceutically acceptable carrier and the DNA encoding VapA or an immunogenic fragment thereof.

15. The method of claim 14 wherein the vaccine is administered by injection.

16. The method of claim 15 wherein the vaccine is injected into muscle or skin.

17. The method of claim 16 wherein the vaccine is injected into muscle.

18. The method of claim 14 wherein the vaccine is administered by a gene gun.

19. The method of claim 18 wherein the vaccine is administered into skin.

20. The method of claim 1 wherein the vertebrate is a mammal.

21. The method of claim 20 wherein the mammal is selected from the group consisting of pigs, horses, and humans.

22. The method of claim 21 wherein the mammal is a horse.

23. The method of claim 22 wherein the mammal is a foal.

24. A composition comprising a pharmaceutically acceptable carrier and recombinant bacteria which express DNA encoding VapA or an immunogenic fragment thereof.

25. The composition of claim 24 wherein the DNA encoding VapA or an immunogenic fragment thereof is extrachromosomal.

26. The composition of claim 24 wherein the DNA encoding VapA or an immunogenic fragment thereof is integrated into the bacterial genome.

27. The composition of claim 24 wherein the bacteria are an avirulent strain of Rhodococcus.

28. The composition of claim 27 wherein the bacteria are an avirulent strain of *Rhodococcus equi*.

29. The composition of claim 28 wherein the DNA encoding VapA or an immunogenic fragment thereof is extrachromosomal.

30. The composition of claim 28 wherein the DNA encoding VapA or an immunogenic fragment thereof is integrated into the bacterial genome.

31. The composition of claim 24 wherein the bacteria are Mycobacteria.

32. The composition of claim 31 wherein the bacteria are BCG.

33. The composition of claim 24 wherein the bacteria also express an immunostimulatory cytokine.

34. A method for expressing a recombinant nucleic acid of interest in Rhodococcus cells comprising:

(1) providing a recombinant vector which comprises the nucleic acid of interest and a mycobacterial origin of replication;

(2) transforming the Rhodococcus cells with said recombinant vector; and

(3) culturing the transformed Rhodococcus cells under conditions wherein the nucleic acid of interest is expressed.

35. The method of claim 34 wherein the nucleic acid of interest is operatively linked to regulatory sequences.

36. The method of claim 35 wherein the vector further comprises an *E. coli* origin of replication.

37. The method of claim 34 wherein the recombinant nucleic acid encodes a polypeptide of interest.

38. The method of claim 37 further comprising recovering the polypeptide from the culture of transformed cells.

39. A method of inducing an immune response in a vertebrate comprising administering recombinant Rhodococcus bacteria which express DNA encoding an immunogen.

40. The method of claim 39 wherein the DNA encoding the immunogen is extrachromosomal.

41. The method of claim 39 wherein the DNA encoding the immunogen is integrated into the bacterial genome.

42. The method of claim 39 wherein the bacteria also express an immunostimulatory cytokine.

43. The method of claim 39 wherein the bacteria are *R. equi.*

44. A method of preventing Rhodococcus infection in a vertebrate comprising administering a vaccine to the vertebrate, wherein said vaccine comprises a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof.

45. A method of preventing pneumonia associated with Rhodococcus infection in a vertebrate comprising administering a vaccine to the vertebrate, wherein said vaccine comprises a nucleic acid encoding the *R. equi* VapA protein or an immunogenic fragment thereof.

46. The method of claim 45 wherein said vertebrate is a horse.

47. The method of claim 46 wherein said vertebrate is a foal.

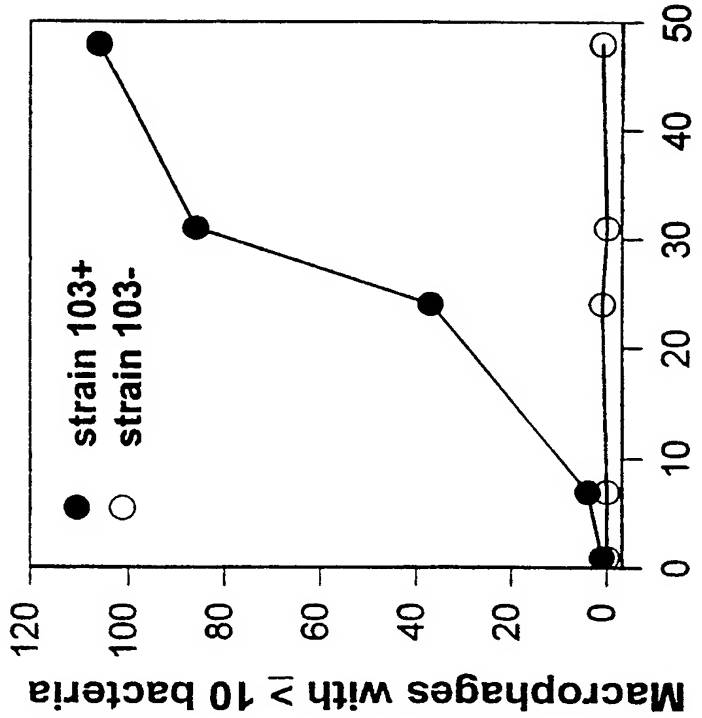

Macrophages with ≥ 10 bacteria
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)
strain 103+
strain 103-

FIG. 1B

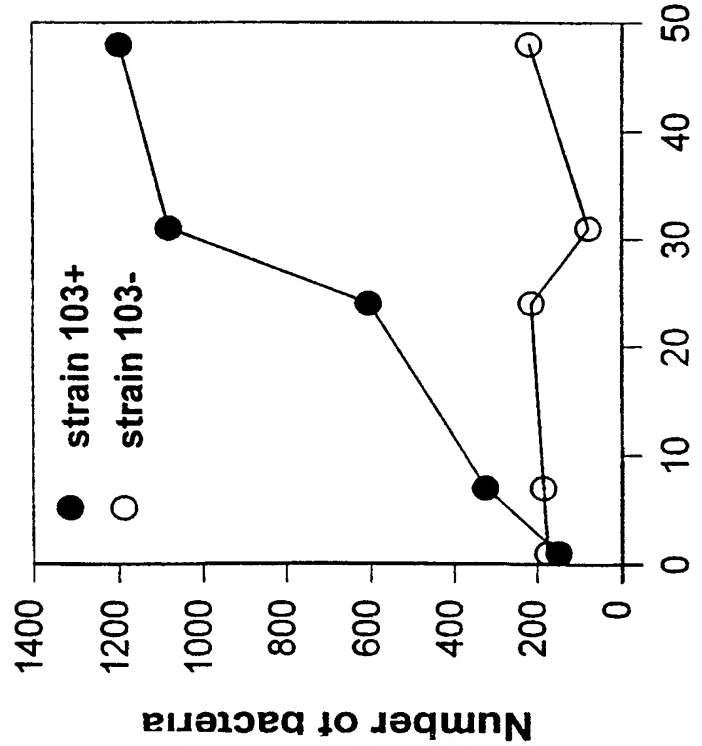

Number of bacteria
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Time (h)
strain 103+
strain 103-

FIG. 1A

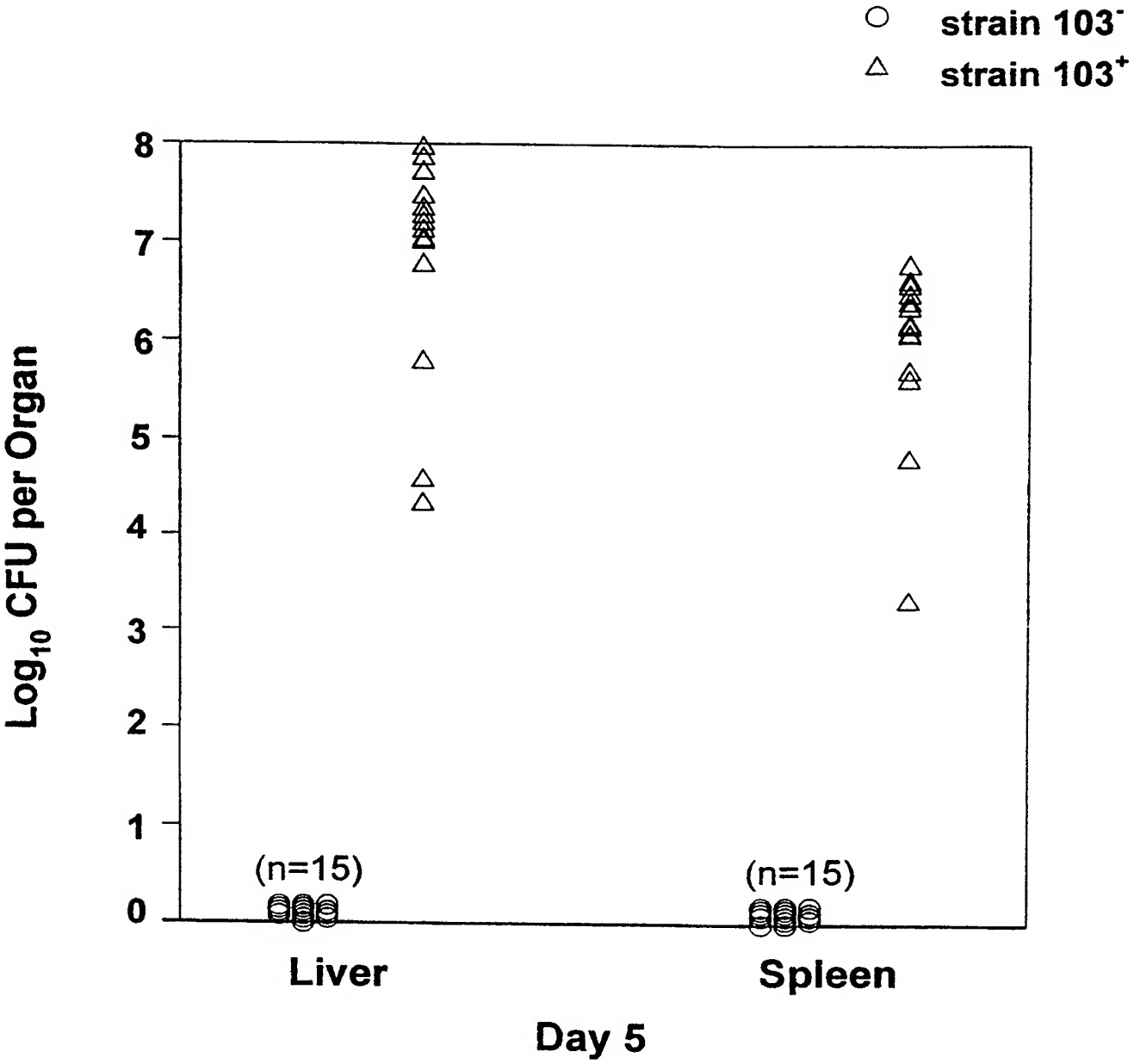
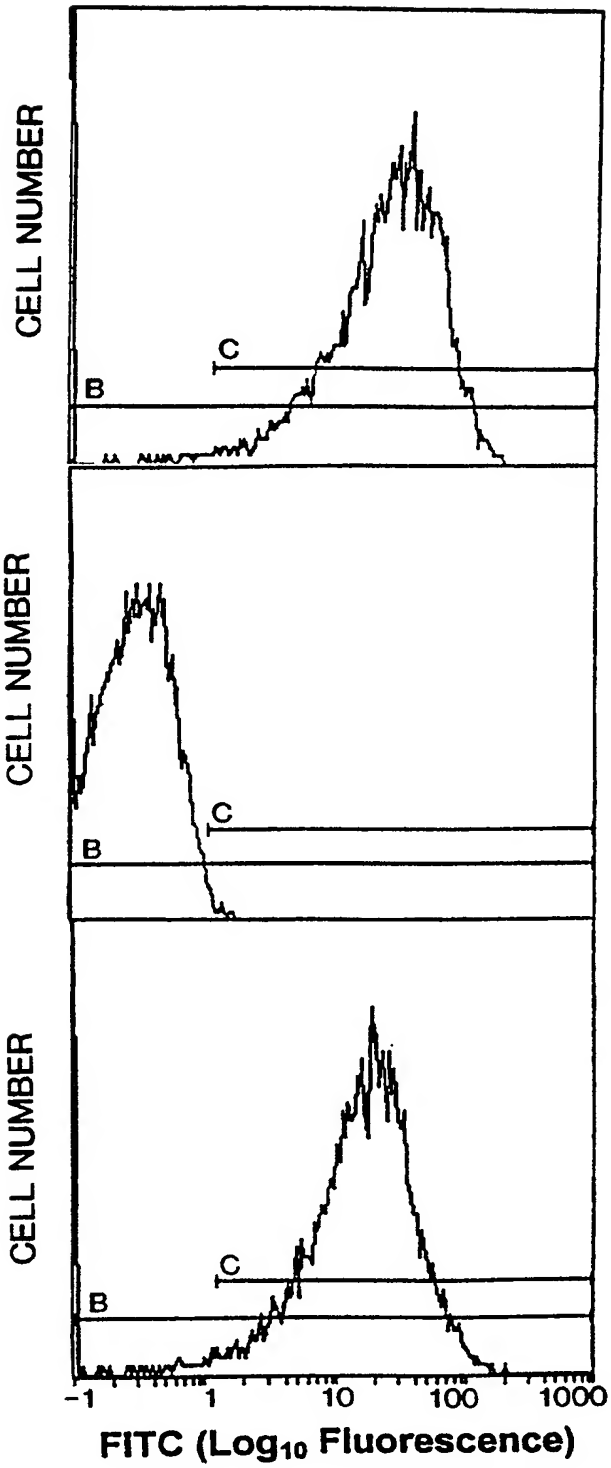

strain 103-
strain 103+
Log10 CFU per Organ
8
7
6
5
4
3
2
1
0
(n=15)
(n=15)
Liver
Spleen
Day 5

FIG. 2

FIG. 3A

FIG. 3B

FIG. 3C



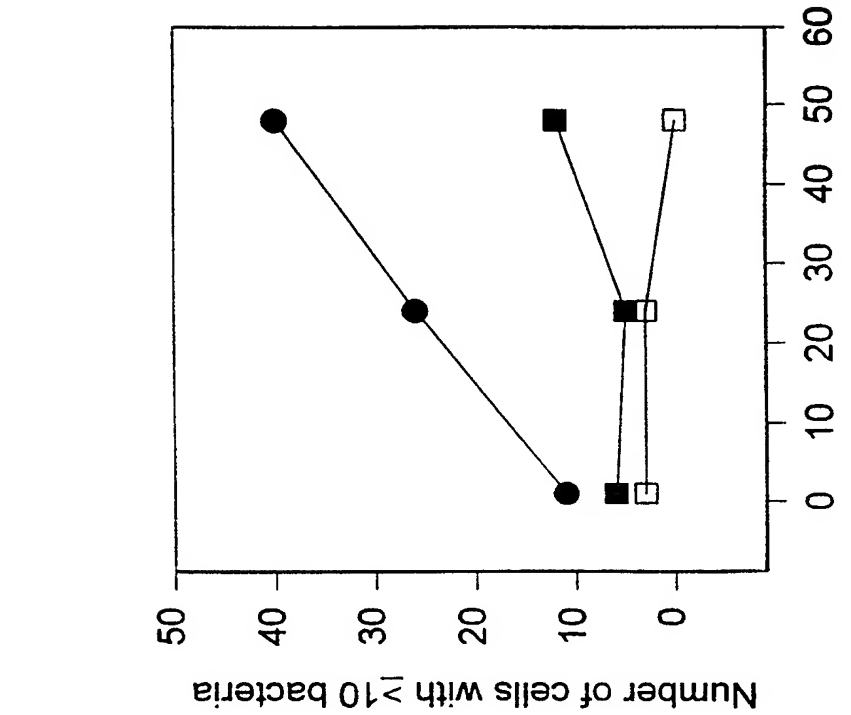

Number of cells with ≥10 bacteria
0
10
20
30
40
50
0
10
20
30
40
50
60
time (hours)

FIG. 4B

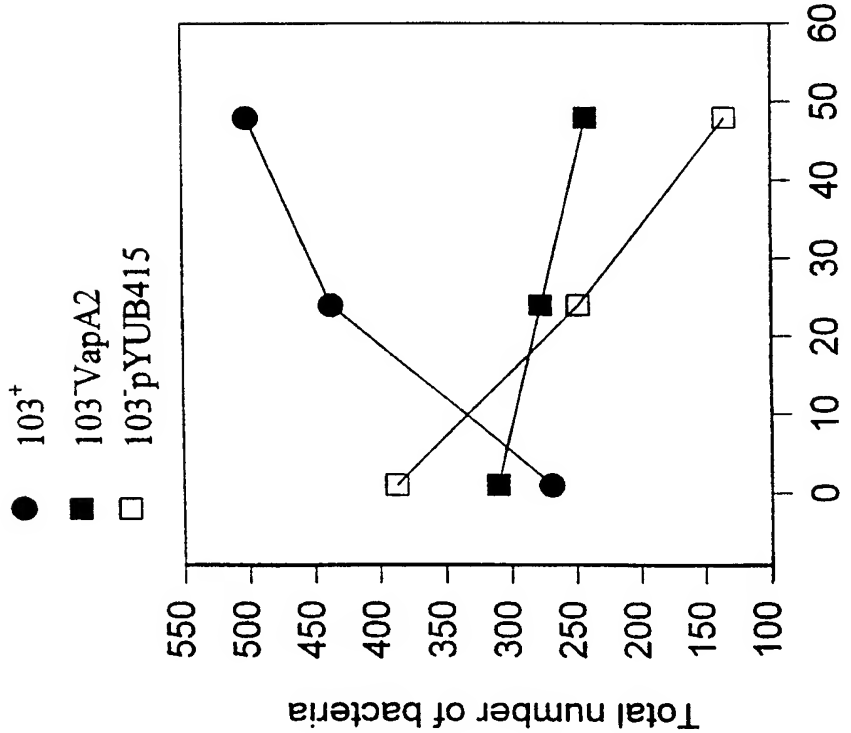

Total number of bacteria
100
150
200
250
300
350
400
450
500
550
0
10
20
30
40
50
60
time (hours)
103+
103-VapA2
103-pYUB415

FIG. 4A

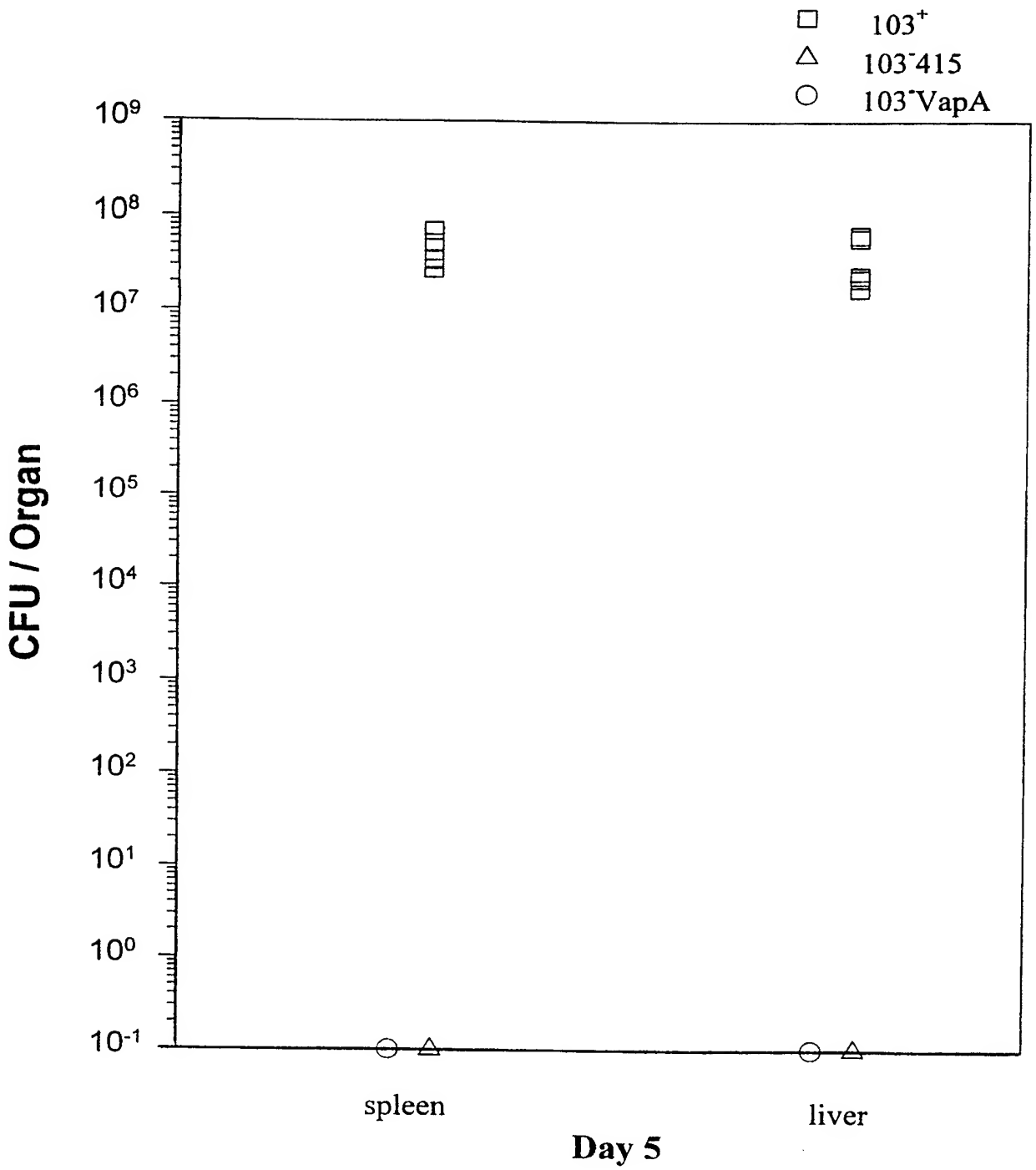

103+
103-415
103-VapA
CFU / Organ
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
spleen
liver
Day 5

FIG. 5

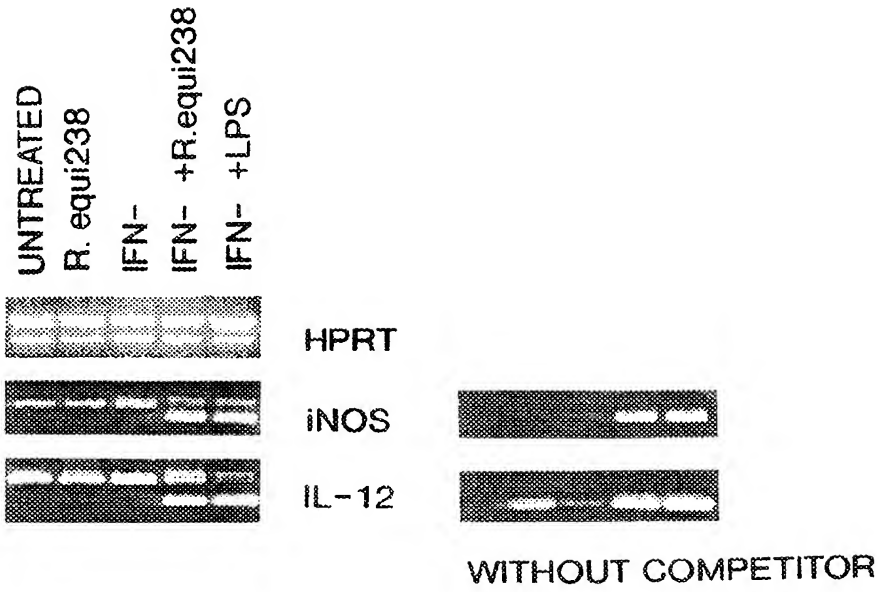

UNTREATED
R. equi238
IFN-
IFN- +R.equi238
IFN- +LPS
HPRT
iNOS
IL-12
WITHOUT COMPETITOR

FIG. 6

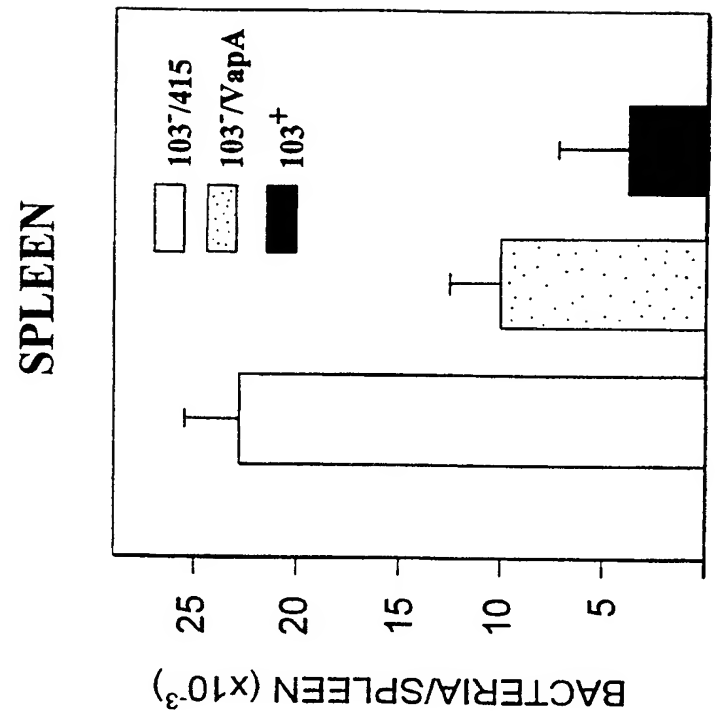

SPLEEN
103⁻/415
103⁻/VapA
103⁺
25
20
15
10
5
BACTERIA/SPLEEN (x10⁻³)

FIG. 7B

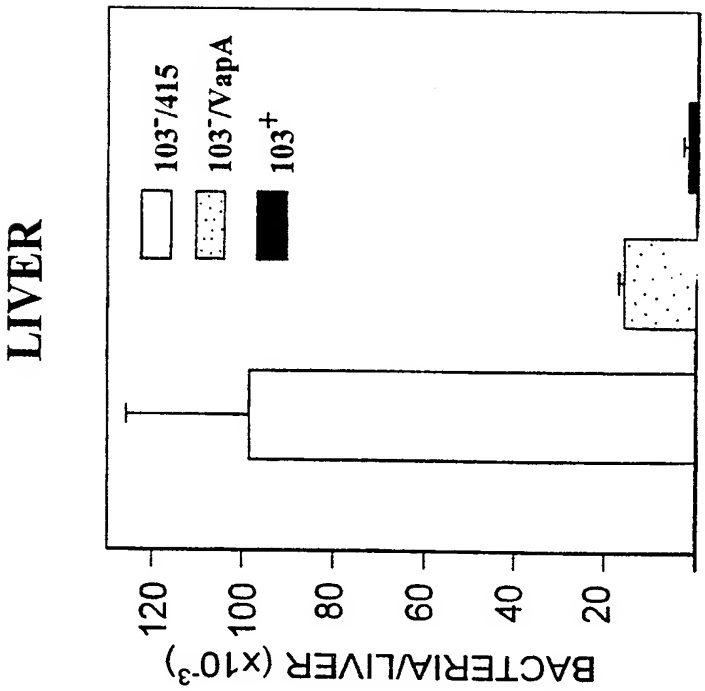

LIVER
103⁻/415
103⁻/VapA
103⁺
120
100
80
60
40
20
BACTERIA/LIVER (x10⁻³)

FIG. 7A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15911

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 172.3, 69.1, 7.2, 375, 325, 243, 252.1; 424/93.1, 93.21; 514/44, 2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, EMBASE, BIOSIS
search terms: VAP A, Rhodococcus, equi, nucleic acid, treatment, gene therapy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	ANZAI et al. Comparison of tracheal aspiration with other tests for diagnosis of Rhodococcus equi pneumonia in foals. Veterinary Microbiology. 16 June 1997, Vol. 56, No. 3-4, pages 335-345, see entire article.	24, 25 1-23, 26-47
Y	BECU et al. Immunoprophylaxis of Rhodococcus equi pneumonia in foals. Veterinary Microbiology. 16 June 1997, Vol. 56, No. 3-4, pages 193-204, see entire article.	1-47
X Y	HAITES et al. Prevalence of the virulence-associated gene of Rhodococcus equi in isolates from infected foals. J. of Clinical Microbiology. June 1997, Vol. 35, No. 6, pages 1642-1644, see entire article.	24, 25 1-23, 26-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
13 OCTOBER 1998

Date of mailing of the international search report
27 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
KAREN M. HAUDA
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15911

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	HONDALUS, M.K. Pathogenesis and virulence of Rhodococcus equi. Veterinary Microbiology. 16 June 1997, Vol. 56, No. 3-4, pages 257-268, see entire article.	24, 25 1-23, 26-47
Y	SUTCLIFFE, I.C. Macroamphiphilic cell envelope components of Rhodococcus equi and closely related bacteria. Veterinary Microbiology. 16 June 1997, Vol. 56, No. 3-4, pages 287-299.	1-47
X Y	PRESCOTT et al. Assessment of the immunogenic potential of Rhodococcus equi virulence associated protein (VapA) in mice. Veterinary Microbiology. Vol. 56, No. 3-4, pages 213-225, see entire article.	1-3, 14, 15, 20 5-13, 16-19, 21-47
Y	PRESCOTT et al. Use of Rhodococcus equi virulence-associated protein for immunization of foals against R. equi pneumonia. American J. of Veterinary Research. April 1997, Vol. 58, No. 4, pages 356-359, see entire article.	1-47
Y	TAN et al. Molecular characterization of a lipid-modified virulence-associated protein of rhodococcus equi and its potential in protective immunity. Canadian J. of Veterinary Research. January 1995, Vol. 59, No. 1, pages 51-59, see entire article.	1-47
Y	FERNANDEZ et al. Protective effect against Rhodococcus equi infection in mice of IgG purified from horses vaccinated with virulence associated protein (VapA)-enriched antigens. Veterinary Microbiology. 16 June 1997, Vol. 56, No. 3-4, pages 187-192, see entire article.	1-47
Y	NICHOLSON et al. Restriction enzyme analysis of the virulence plasmids of VapA-positive Rhodococcus equi strains isolated from humans and horses. J. of Clinical Microbiology. March 1997, Vol. 35, No. 3, pages 738-740, see entire article.	1-47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15911

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C12N 21/00, 15/63, 15/79, 15/00, 15/11, 15/09, 5/10; A61K 48/00, 38/00, 39/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL : 435/320.1, 172.3, 69.1, 7.2, 375, 325, 243, 252.1; 424/93.1, 93.21; 514/44, 2